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Synthesis of Repressible Acid Phosphatase in *Saccharomyces cerevisiae* Under Conditions of Enzyme Instability

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The synthesis of repressible acid phosphatase in *Saccharomyces cerevisiae* was examined under conditions of blocked derepression as described by Toh-e et al. (Mol. Gen. Genet. 162:139–149, 1978). Based on a genetic and biochemical analysis of the phenomenon these authors proposed a new regulatory model for acid phosphatase expression involving a simultaneous interaction of regulatory factors in the control of structural gene transcription. We demonstrate here that under growth conditions that fail to produce acid phosphatase the enzyme is readily inactivated. Furthermore, we demonstrate under these conditions the production of acid phosphatase mRNA which is active both in vitro and in vivo in the synthesis of enzyme. This eliminates any step prior to translation of acid phosphatase polypeptide as an explanation for the phenomenon. We interpret our results for the block in appearance of acid phosphatase as a result of both deaccelerated growth and cellular biosynthesis during derepression, accompanied by an enhanced instability of the enzyme.

Synthesis of repressible acid phosphatase (APase; orthophosphoric-monoester phosphohydrolase [acid optimum], EC 3.1.3.2) in *Saccharomyces cerevisiae* is negatively controlled by P_i levels present in the growth medium (13). The enzyme is a secreted glycoprotein consisting of as many as four polypeptides (D1 through D4), each approximately 60,000 in molecular weight (6). The repression of enzyme synthesis has been shown by Oshima and colleagues (18–20) to be due to the action of a number of regulatory genes dispersed throughout the yeast genome. They proposed, from genetic evidence, a role for these genes involving transcriptional regulation via the sequential functioning of their products, analogous to control circuits in a bacterial operon (1). Support for transcriptional regulation is provided by a recent demonstration that mRNA's coding for APase polypeptides (p60, p58, and p56) are controlled by P_i (6). However, little else is known about the molecular mechanisms regulating their expression.

Recently, Toh-e et al. (17) observed that when *S. cerevisiae* is grown at a pH below 3.0 in low-P_i medium containing $(NH_4)_2SO_4$ as the nitrogen source, derepression of APase does not occur. Release from the block in derepression is immediate upon shift to high pH, but requires protein synthesis. Toh-e et al. suggested that under these conditions the essential functioning of the regulatory genes for structural gene transcription is disturbed. Through a series of experiments using temperature-sensitive mutants and coupled temperature and pH shifts, these authors proposed a revised model wherein regulatory factors encoded by the numerous regulatory genes control structural gene expression simultaneously by direct molecular interaction. This novel mechanism has also been proposed by a number of investigators for regulatory control in the well-characterized yeast galactose system (10, 11). For APase, at low pH the positive factor in the regulatory aggregate is decomposed or inactivated, leading to a loss of derepression.

A direct prediction of this revised model is that production of APase mRNA would be blocked when cells are grown in low-pH $(NH_4)_2SO_4$ medium, even at low concentrations of P_i. As a consequence, APase polypeptide synthesis and post-translational modification would not be observed. In this paper, we have attempted to directly test these predictions under experimental conditions identical to those employed by Toh-e et al. (17). In the course of the design of these experiments we have found that the stability of exocellular APase at pH 3.0 is substantially influenced by medium composition and is least stable with $(NH_4)_2SO_4$ as the nitrogen source. Although appearance of enzyme activity ceases at the low pH in repeat experiments, mRNA's for APase polypeptides are synthesized and are functional in vitro. Furthermore, by radioimmune assay and by immunoprecipitation and deglycosylation of protein from radiolabeled cell extracts, we have demon-

strated both protein synthesis and post-translational modification of APase in $(NH_4)_2SO_4$ (low pH)-grown cells. Although these data do not directly support either model for the regulatory control of APase synthesis, we have eliminated any step prior to translation as an explanation for the observed phenomenon. We interpret our results as an overall reduction in protein synthesis during derepression by growth on $(NH_4)_2SO_4$ (low-pH) minimal medium, accompanied by an enhanced instability of the enzyme.

MATERIALS AND METHODS

Yeast strains and media. Haploid strain H42 (ATCC 26922; a *PHO3 PHO5*) has the wild-type genes for both the repressible acid and alkaline phosphatase. It also produces a low level of a constitutive APase in synthetic medium (18). The compositions of high-P_i minimal medium (1,500 mg of KH_2PO_4 per liter in modified Burkholder synthetic medium to which L-asparagine was added as the sole source of nitrogen), low-P_i minimal medium (the KH_2PO_4 in high-P_i medium was reduced to 30 mg, and 1,500 mg of KCl per liter was added), and nutrient medium are described elsewhere (18). In some cases, 2 g of $(NH_4)_2SO_4$ per liter was used as a source of nitrogen in the same synthetic medium in place of L-asparagine. SMD medium (pH 4.7) consisted of modified Burkholder minimal medium supplemented with one-fifth strength low-P_i yeast extract-peptone (Difco) (6).

Partial purification of APase. Repressible APase was obtained from strain P28-24C (a *pho3-1 PHO5*) grown in SMD low-P_i medium. Cells were washed once with 0.1 M sodium acetate buffer (pH 4.2), resuspended in 1.5 volumes of buffer, and disrupted with glass beads (4). The enzyme was then purified by the method of Boer and Steyn-Parve (2) with only minor modification. For stability experiments the Sephadex G75 filtrate fraction was used, at a specific activity of 19.5 enzyme units per mg of protein.

APase activity. APase activity was measured directly in whole cell suspensions by a spectrophotometric assay using *p*-nitrophenyl phosphate as a substrate. Reactions containing 2.25 mg of substrate in a total volume of 0.5 ml were run at 37°C in 0.1 M sodium acetate buffer (pH 4.2) and stopped by the addition of 0.12 ml of 25% (wt/vol) trichloroacetic acid and 0.6 ml of saturated Na_2CO_3. Cells were removed by centrifugation, and the absorbance was measured at 420 nm. One unit of enzyme activity was taken as that liberating 1 μmol of *p*-nitrophenol per min.

Cell growth, RNA isolation, and preparation of cell extracts. All yeast cultures used in this work were started with fresh overnight inocula prepared in either high-P_i SMD or high-P_i asparagine minimal medium (at approximately 3×10^8 cells per ml) and added at a cell density of 10^6/ml into either low-P_i or high-P_i medium. Growth was at 30°C with shaking and monitored turbidimetrically as optical density at 660 nm (OD_{660}). Enzyme assays were performed on portions of these cultures which had been supplemented with cycloheximide to 0.05 mg/ml and either transferred to 4°C before the enzyme assay or stored at −80°C. Total cellular RNAs were prepared from cycloheximide-arrested cells by standard water-saturated phenol extraction in the presence of 0.1% (wt/vol) sodium dodecyl sulfate (SDS) after cell disruption with glass beads, as described previously (4, 6). Radiolabeled cell extracts were prepared by pulse-labeling cultures with L-[^{35}S]methionine at an initial radioisotope concentration of 30 μCi/0.0375 pmol per ml for 10 min, followed by a 2-min chase with 1 mM methionine and arrest with 0.05 mg of cycloheximide per ml. Extractions were performed essentially as previously described (6) with 0.1% (wt/vol) Triton X-100 in the extraction buffer. Rates of radioisotope incorporation into protein and of protein-specific activities were determined by trichloroacetic acid precipitation (8) and Lowry protein determination (9).

Cell-free protein synthesis and immunoprecipitation. Total yeast cellular RNAs were translated in a wheat germ cell-free system as previously described (5, 6), with L-[^{35}S]methionine (>800 Ci/mmol; Amersham/Searle) as the incorporated label. All translations were performed under standard conditions in which cell-free synthetic activity was proportional to RNA concentration. Specific mRNA activities were determined by immunoprecipitation of their cell-free products from the total translation reactions by using immune rabbit immunoglobulin G (IgG). For immunoprecipitation of proteins from cell extracts, 1.5×10^6 cpm of radiolabeled protein was used (at specific activities from 2×10^4 to 1.2×10^5 cpm/μg), with total protein adjusted to 1 mg/ml with bovine serum albumin. Immunoprecipitations were performed by the solid-state protein A-Sepharose 4BCL procedure previously described (6). Radiolabeled APase immunoprecipitated from cell extracts with anti-APase IgG was deglycosylated by endoglycosidase H digestion after dissociation from the protein A-Sepharose in 0.1 M Tris (pH 8.0)–1% (wt/vol) SDS–1% (wt/vol) β-mercaptoethanol at 100°C, followed by treatment with iodoacetamide (12). Immunoprecipitates were analyzed on SDS-polyacrylamide slab gels by electrophoresis and autoradiography, after PPO (2,5-diphenyloxazole) impregnation by published methods (5, 12). Densitometry of gel autoradiograms was done with a Joyce-Loebl densitometer. Radioimmune assays were preformed by a modification of the protein A-Sepharose 4BCL procedure (6) using radioiodinated APase, purified as previously described (6). Cell extracts were prepared as described above, and protein concentrations were determined by the method of Lowry et al. (9). For each assay 6 μg of protein from a cell extract or nonradioactive APase covering a 50-fold concentration range was added 5 min before addition of the radioiodinated antigen, and the radioactivity of the immunoprecipitated protein was determined. APase concentrations in the cell extracts were determined from a standard curve plot. No reduction in immunoprecipitation was observed in control assays for samples taken from similar cultures grown under repressed (high-P_i) conditions.

RESULTS

Effect of nitrogen source on APase derepression. Previously, Toh-e et al. (17) demonstrated that when strain H42 (*PHO3 PHO5*) is grown in a low-P_i minimal medium with L-asparagine as a sole nitrogen source, derepression of APase occurs at cell densities of about 10^7/ml. Howev-

er, when H42 is grown with $(NH_4)_2SO_4$ as the sole nitrogen source, no derepression occurs. A similar experiment (Fig. 1) confirmed their observation. In low-P_i minimal medium containing L-asparagine [or both L-asparagine and $(NH_4)_2SO_4$; data not shown], APase derepression was observed when the growth exceeded an OD_{660} of ca. 0.5. Substitution of $(NH_4)_2SO_4$ as the nitrogen source had no effect on the initial growth rate but dramatically reduced the level of APase derepression. The final pH values of the cultures in Fig. 1 were 4.2 for asparagine minimal medium and 2.5 for $(NH_4)_2SO_4$ minimal medium, consistent with the findings of Toh-e et al. (17).

Effect of pH and medium on APase derepression. To characterize the phenomenon of pH and medium effects on APase derepression (illustrated in Fig. 1), a series of experiments were performed to determine whether the observed differences in pH had a direct effect on derepression, or whether other factors were involved. The high levels of APase synthesized in low-P_i asparagine minimal medium (pH 4.7) (Fig. 1) provided an opportunity to test this. At intervals during growth of H42 in this medium, the pH was decreased to 3.0 by the addition of 0.4 N HCl, and the derepression of APase was followed. As shown in Fig. 2, decreasing the pH from 4.7 to 3.0 delayed but did not prohibit APase synthesis. Derepression occurred regardless of the pH shift at all times throughout the growth phase of the culture. We therefore repeated the experiment of Fig. 1 with media buffered with 0.05 M sodium citrate to a constant pH (pH 3.0 or 4.7). Cells were inoculated into low-P_i medium at 10^6 cells per ml and aerated at 30°C (Fig. 3). In buffered minimal medium with asparagine as the sole nitrogen source, the growth rates and levels of derepression at pH 3.0 and 4.7 were essentially the same. There was a slight delay in derepression at pH 3.0, consistent with that observed in the experiment of Fig. 2. In minimal medium containing $(NH_4)_2SO_4$ as the sole nitrogen source, the growth rates at both pH 4.7 and 3.0 were substantially reduced. At pH 4.7 derepression of APase occurred at slightly higher culture densities than in asparagine minimal medium, but the same maximal enzyme levels were achieved by stationary phase. However, at pH 3.0, whereas derepression occurred at the same growth stage as the pH 4.7 medium, the amount of derepression was severely reduced. This reduced level of enzyme activity was higher than in the unbuffered $(NH_4)_2SO_4$ minimal medium in the experiment of Fig. 1, probably reflecting the higher adjusted pH. The data, however, indicate that it is not pH per se which affects APase derepression, but rather a combined effect of pH and

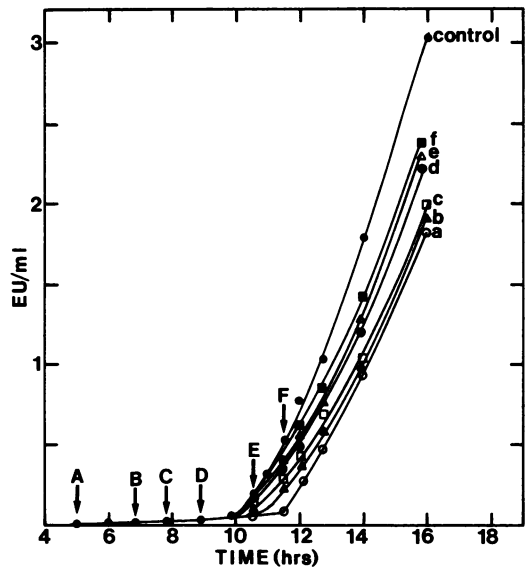

FIG. 2. Effect of pH on APase derepression low-P_i asparagine minimal medium. H42 was grown as shown in Fig. 1 in low-P_i asparagine minimal medium. At intervals as indicated by arrows (A through F), the pH was decreased to 3.0 by the addition of 0.4 N HCl, and derepression of APase was followed during growth at 30°C. Plots a through f show the enzyme activity with time for cultures treated as indicated by the corresponding arrows.

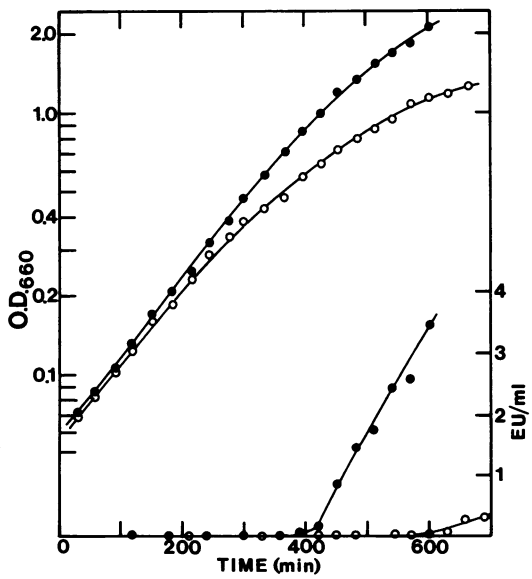

FIG. 1. Effect of nitrogen source on APase derepression. Cells of H42 were grown at 30°C in low-P_i minimal medium containing either L-asparagine (●) or $(NH_4)_2SO_4$ (○) as the sole nitrogen source. Upper curves: Growth as measured by OD_{660}; lower curves: units of APase per milliliter.

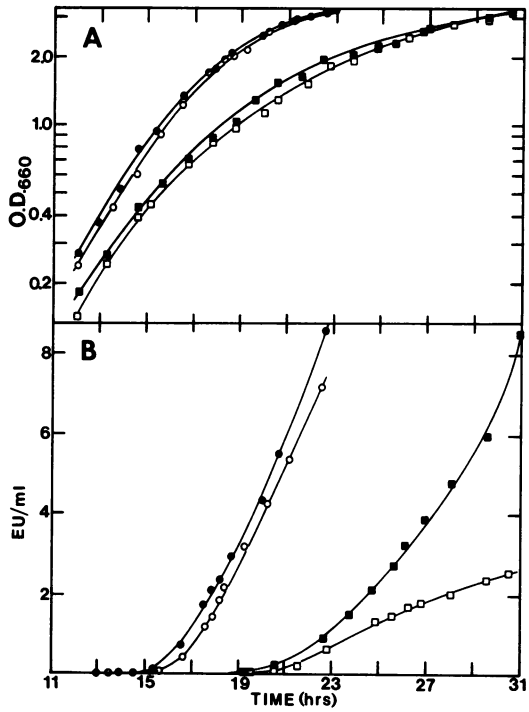

FIG. 3. Growth and APase derepression in buffered minimal medium. H42 was grown in low-P_i minimal medium buffered with 0.05 M citrate buffer: asparagine minimal medium at pH 4.7 (●) or pH 3.0 (○); $(NH_4)_2SO_4$ minimal medium at pH 4.7 (■) or pH 3.0 (□). (A) Growth as measured by OD_{660}; (B) units of APase per ml.

condition of growth. In fact, in SMD medium, in which growth and production of APase are dependent upon complex organic phosphates from low-P_i yeast extract-peptone (6) and which contains both asparagine and $(NH_4)_2SO_4$, derepression was not dramatically reduced by lowering the pH to 3.0, in spite of the presence of $(NH_4)_2SO_4$ in the medium.

Transcription of APase mRNA during growth at low pH in $(NH_4)_2SO_4$ minimal medium. The original interpretation by Toh-e et al. (17) for the block in APase derepression was that at the low culture pH of the $(NH_4)_2SO_4$ minimal medium the normal positive control of APase structural gene transcription is disturbed, such that APase is not synthesized. We have clearly demonstrated above that pH alone, at least above 3.0, is not solely responsible for the phenomenon. To directly test their model on the mechanism for this block in derepression, we assayed for APase structural gene transcription by measuring the in vitro activities of APase mRNA's, as previously described (6). The data are shown in the autoradiogram of Fig. 4. In low-P_i asparagine minimal medium, three mRNA activities for the in vitro APase polypeptides p60, p58, and p56 accumulated in cells during the later stages of growth, slightly preceding enzyme derepression. In low-P_i $(NH_4)_2SO_4$ minimal medium, where no net accumulation of enzyme activity occurred, all three APase mRNA's were synthesized at significant levels (Fig. 4B). In fact, the ratio of RNA to enzyme activity in the $(NH_4)_2SO_4$ growth condition was at least 20-fold greater than that observed for the asparagine culture. A limitation, however, to using translatability to make comparative measurements of APase gene transcription between the two cultures, besides the variation in extraction and survival of mRNA during preparation, is the decrease in mRNA synthesis with declining growth rate and the lower translatability of mRNA isolated from cells entering stationary phase of growth. Under both of these growth conditions assimilation of the same amount of the supplied inorganic phosphate should be required for derepression of APase, as there is an absolute requirement of phosphate for growth and derepression of enzyme (13, 15). We have demonstrated elsewhere that growth yield in different media is related sigmoidally with the logarithm of the concentration of supplied phosphate (Bostian, Lemire, and Halvorson, unpublished data) such that comparisons of APase levels (or mRNA levels) should be made at equivalent culture densities. However, in this experiment at equivalent growth yields the cultures were considerably different. In $(NH_4)_2SO_4$ minimal medium the culture was entering stationary phase earlier and more slowly than in the asparagine minimal medium (Fig. 4A). To overcome some of these difficulties in comparisons of mRNA levels at equivalent growth yields (OD_{660}), we assayed enolase mRNA as an internal control. From experiments in a variety of media and at different exponential growth rates, we have determined that the levels of mRNA and polypeptide for this constitutive glycolytic enzyme remain a fairly constant proportion of the total cellular mRNA or protein (5, 6; Bostian, Lemire, and Halvorson, unpublished data). In Fig. 4B we have normalized the APase mRNA levels of the Fig. 4 autoradiogram to that for enolase. As seen, the normalized densitometer tracings of APase mRNA levels were approximately the same in both cultures at equivalent growth yields. For example, at an OD_{660} of ca. 1.3 the ratios of p60 APase to enolase translatable mRNA were 0.30 and 0.46 for the low-P_i asparagine and $(NH_4)_2SO_4$ minimal medium cultures, respectively. Thus at low pH in low-P_i $(NH_4)_2SO_4$ minimal medium APase mRNA continues to be synthesized and is functional in vitro in the synthesis of APase polypeptides.

Direct measurement of APase synthesis. The

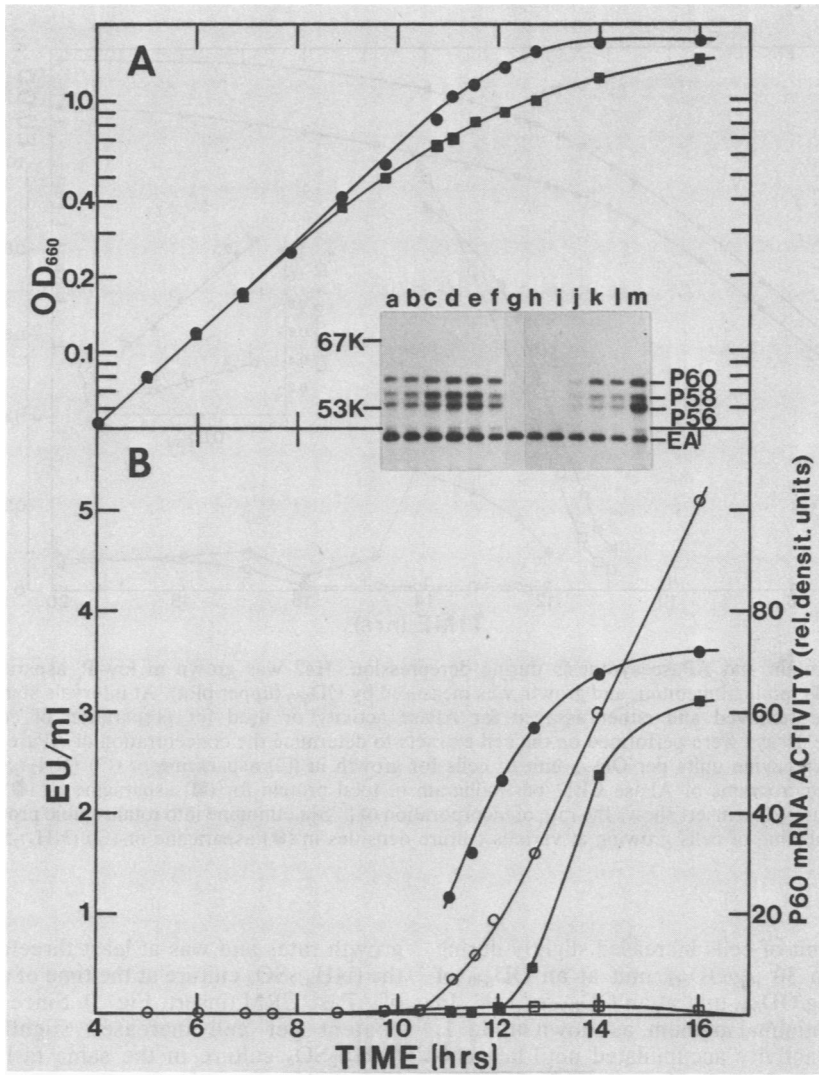

FIG. 4. Accumulation of APase mRNA during growth at low pH. H42 was grown in low-P_i asparagine (●) or $(NH_4)_2SO_4$ (■) minimal medium. At intervals starting at 11 h, samples were removed from the asparagine (lanes a through f) and the $(NH_4)_2SO_4$ (lanes g through l) cultures and assayed for APase and enolase mRNA by in vitro translation and immunoprecipitation, followed by electrophoresis on a 10% SDS-polyacrylamide slab gel and autoradiography, as described in the text. Lane m is a control showing the immunoprecipitated in vitro translation products of RNA isolated from strain 28-24C grown to an OD_{660} of 1.0 in SMD low-P_i medium. (A) Growth measured by OD_{660}; (B) units of APase per milliliter (open symbols) and relative densitometer density units of p60 synthesized in vitro (closed symbols) (from gel autoradiogram insert), normalized to the corresponding in vitro-synthesized enolase.

production of APase mRNA but not active enzyme in the above experiments might be explained in several ways: by a disruption in some translational step in vivo that leads to enzyme synthesis, by some post-translational modification involved in enzyme formation (glycosylation, transport, etc.), or by enzyme turnover. To test these possibilities we directly determined the amount of in vivo accumulated APase polypeptide under the blocked derepression conditions by radioimmune assay. Cells of H42 were grown in asparagine or $(NH_4)_2SO_4$ minimal media with 1/50 P_i, and at various times samples were removed for enzyme assay and for preparation of cell extracts. The soluble fraction was taken for measurement of protein content and for radioimmune assay (Fig. 5). An approximately parallel increase in APase activity and polypeptide accumulation occurred for growth in asparagine minimal medium. Protein content

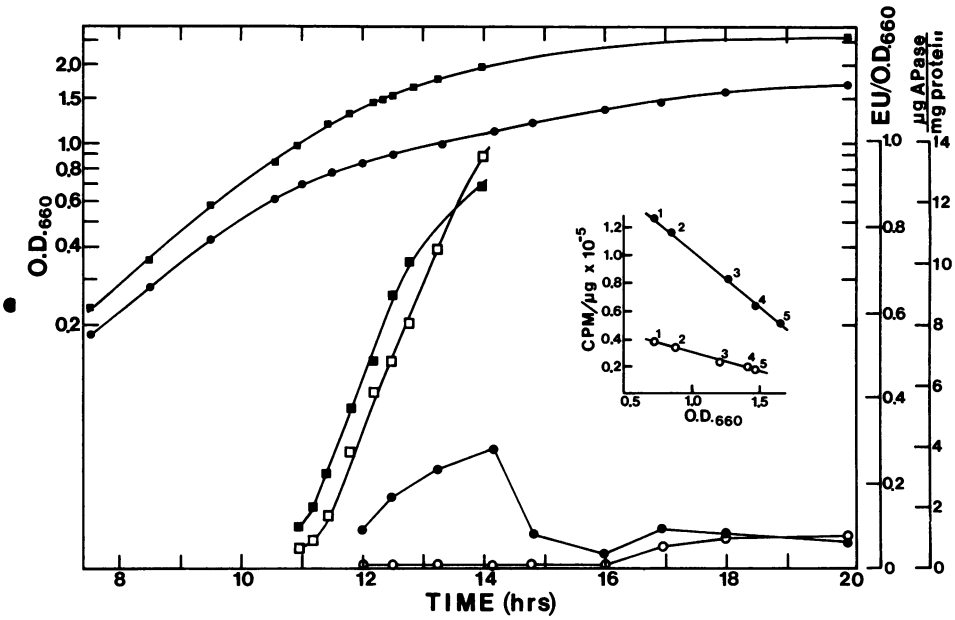

FIG. 5. Protein and APase synthesis during derepression. H42 was grown in low-P_i asparagine (■) or $(NH_4)_2SO_4$ (●) minimal medium, and growth was measured by OD_{660} (upper plot). At intervals starting at 11 h, samples were removed and either assayed for APase activity or used for preparation of cell extracts. Radioimmune assays were performed on the cell extracts to determine the concentration of APase CRM. Data are shown as enzyme units per OD_{660} unit of cells for growth in (□) asparagine or (○) $(NH_4)_2SO_4$ minimal medium, or micrograms of APase CRM per milligram of total protein for (■) asparagine or (●) $(NH_4)_2SO_4$ minimal medium. The insert shows the rate of incorporation of [^{35}S]methionine into total soluble protein during a short pulse-labeling of cells growing at various culture densities in (●) asparagine or (○) $(NH_4)_2SO_4$ minimal medium.

per OD_{660} unit of cells increased slightly during growth from 30 μg/OD_{660} unit at an OD_{660} of 0.88 to 39 μg/OD_{660} unit at an OD_{660} of 1.98. In $(NH_4)_2SO_4$ minimal medium, as shown in Fig. 1, no enzyme activity accumulated until late culture densities, when less than 7% of the maximal activity for growth in the asparagine minimal medium occurred. However, there was a substantial initial accumulation of antigenic polypeptide which reached 25% of the maximal level attained in the asparagine culture. The initial appearance of polypeptide occurred at an apparently similar or faster rate than for cells grown in the asparagine medium. For example, at a culture density (OD_{660}) of 1.15 the specific concentrations of APase protein were 3.6 and 2.9 μg of APase cross-reacting material (CRM) per mg of protein, respectively, for the $(NH_4)_2SO_4$ and asparagine cultures. However, beyond this initial synthesis, net accumulations of APase CRM declined with further growth, followed at late culture densities by a slight increase corresponding to a slight increase in enzyme activity. In both cultures the rate of protein synthesis, measured by radioisotope incorporation into protein during a pulse-chase, declined with decreasing growth rates and was at least threefold less for the $(NH_4)_2SO_4$ culture at the time of appearance of APase CRM (insert, Fig. 5). Since the protein content per cell increased slightly for the $(NH_4)_2SO_4$ culture in the same fashion as for growth in asparagine medium, the decline in APase CRM presumably represents turnover and degradation of protein, possibly accompanied by residual polypeptide synthesis. This might be explained by enzyme instability upon decreasing pH during growth in the $(NH_4)_2SO_4$ minimal medium.

The CRM detected by the radioimmune assay appears to represent normal matured APase (Fig. 6). The extracts from [^{35}S]methionine-pulse-labeled cells were immunoprecipitated with APase antibody, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. High-molecular-weight glycosylated APase was readily identified for cells grown in both media. Further characterization of the glycoprotein was made by deglycosylating the immunoprecipitates by endoglycosidase H treatment before electrophoresis. As shown, all four previously characterized APase polypeptides (D1 through D4) were present in immuno-

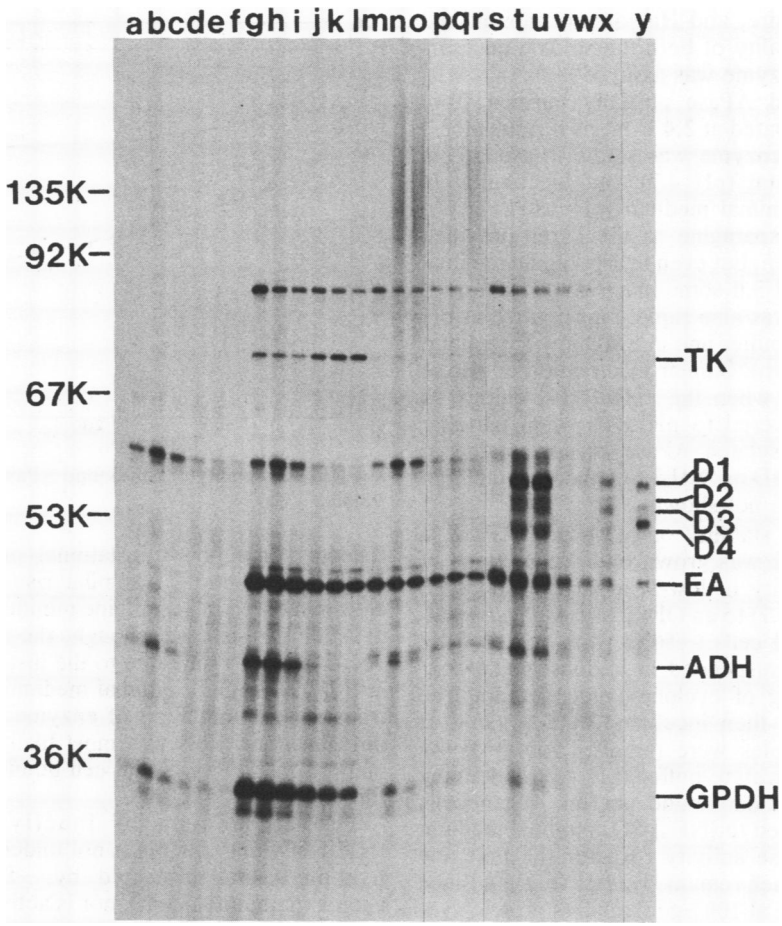

FIG. 6. Autoradiogram of immunoprecipitated and deglycosylated [^{35}S]methionine-labeled APase. The [^{35}S]methionine-labeled cell extracts prepared from pulse-labeled cells growing in asparagine or $(NH_4)_2SO_4$ minimal medium (see insert in Fig. 5) were analyzed by immunoprecipitation with various yeast protein antibodies, followed by electrophoresis on a 10% SDS-polyacrylamide slab gel and autoradiography. Pulse-labeled samples 3 to 5 were immunoprecipitated consecutively with preimmune IgG for cells grown with asparagine (a to c) or $(NH_4)_2SO_4$ (d to f); with a mixture of transketolase (TK), enolase (EA), alcohol dehydrogenase (ADH), and glyceraldehyde 3-phosphate dehydrogenase (GPDH) IgG for cells grown with asparagine (g to i) or $(NH_4)_2SO_4$ (j to l); with APase IgG for cells grown with asparagine (m to o) or $(NH_4)_2SO_4$ (p to r). Lanes s through u are deglycosylated APase IgG immunoprecipitates for cells grown with asparagine (m to o). Lanes v to x are deglycosylated APase IgG immunoprecipitates for cells grown with $(NH_4)_2SO_4$ (p to r). Lane y is an immunoprecipitate with APase IgG of the in vitro translation products of RNA from derepressed cells of strain P28-24C. D1 through D4 represent the deglycosylated separate polypeptides previously characterized for APase (6). Glycosylated APase polypeptides (lanes n, o, q, and r) migrate as a diffuse high-molecular-weight band (ca. 135×10^3).

precipitates from both cell extracts. In these immunoprecipitations enolase antibody was included as a comparative, internal control. It can be seen that its level reflects the overall decline in biosynthetic rate (insert, Fig. 5). Thus, these experiments (Fig. 5 and 6) show a significant accumulation and turnover of glycosylated APase polypeptide under the growth condition of blocked derepression.

Inactivation of APase. Based on the above results, we examined the stability of APase under the various growth conditions employed. Initially, Heredia et al. (7) reported an inactivation of APase by acid treatment. However, Toh-e et al. (17) reported only a 6% loss in enzyme activity after a 2-h treatment at pH 3.0 or 2.8 in asparagine minimal medium. Stability in $(NH_4)_2SO_4$ minimal medium was not examined. Since approximately 15% of APase in H42 is released into the medium during derepression

(Bostian, Lemire, and Halvorson, unpublished data), the stability of both the soluble and surface-bound enzyme was assayed.

Glycosylated APase partially purified from H42 was incubated at 2.4 U/ml in various media at 30°C. The enzyme was stable in asparagine minimal medium (pH 4.7), but inactivated in $(NH_4)_2SO_4$ minimal medium (pH 3.0) (Fig. 7). Addition of asparagine to the latter provided some protection, but even in asparagine minimal medium at pH 3.0 some inactivation occurred. The enzyme was also rapidly inactivated at pH 3.0 in acetate buffer and in SMD low-P_i medium (data not shown). Similar inactivation of APase was observed when the pH of H42 culture in SMD was lowered to pH 3 or when partially purified glycosylated APase was incubated in either used SMD or $(NH_4)_2SO_4$ minimal medium at pH 3.0 (data not shown).

To test the stability of cell-bound APase, a culture of H42 was grown under conditions of enzyme stability in low-P_i asparagine minimal medium (pH 4.7) to an OD_{660} of 1.4. The partially derepressed cells (~50%) were collected by filtration and suspended in various media containing 100 μg of cycloheximide per ml. The cultures were then incubated at 30°C, and at intervals samples were removed for assay by dilution into acetate buffer (pH 4.2) containing 100 μg of cycloheximide per ml. When cells were transferred to $(NH_4)_2SO_4$ minimal medium (pH 4.7) APase activity rose for the first few minutes and then remained stable (Fig. 8). Since cycloheximide at 100 μg/ml inhibits protein synthesis almost immediately, this rise probably

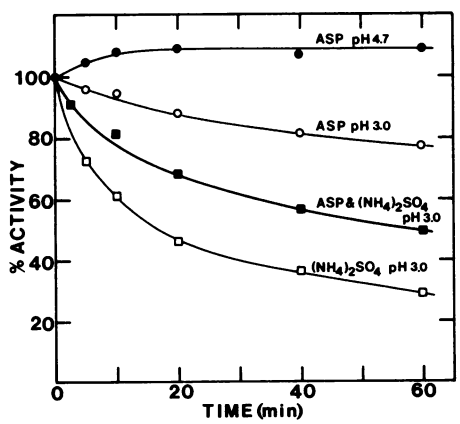

FIG. 7. Inactivation of purified APase by acid treatment. Purified APase from H42 (2.4 U/ml) was incubated at 30°C in various growth media at pH 4.7 and 3.0. At intervals, 0.02-ml samples were diluted with 0.38 ml of assay buffer (pH 4.2), and stored on ice until used for enzyme assay. Percent initial activity was calculated as a function of incubation time.

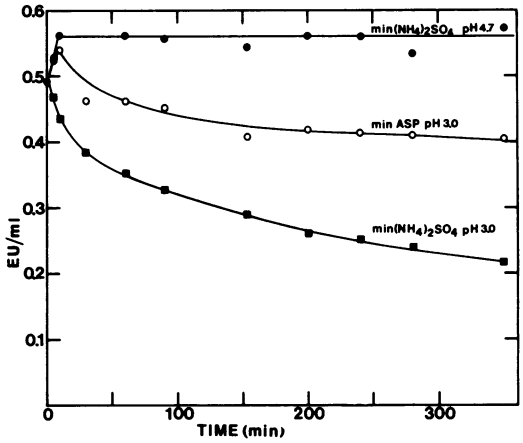

FIG. 8. Stability of cell-bound APase. See text for details.

reflects some post-translational processing of APase polypeptide. A similar rise was seen in cells incubated in asparagine minimal medium at pH 3.0. In this case, though, the activity then decreased with time due to the instability at pH 3.0. In $(NH_4)_2SO_4$ minimal medium at pH 3.0 a transient accumulation of enzyme activity was not observed. This was most likely due to the marked instability of the cell-bound enzyme in this medium at pH 3.0.

The mechanism of inactivation with $(NH_4)_2SO_4$ and low pH is not understood. Inactivation is not protected by −SH reducing agents (data not shown), nor is activity regained upon raising the pH. Furthermore, the kinetics of decay of soluble and cell-bound enzyme (Fig. 9) are complex and suggest more than one component. Cell-bound enzyme is more stable than soluble enzyme, possibly reflecting a more protected state on the cell surface.

DISCUSSION

This study was undertaken to analyze the regulatory mechanism for APase synthesis in *S. cerevisiae* by examining the details of enzyme production under conditions of blocked derepression. Toh-e et al. (17) demonstrated that yeast cells grown in low-P_i synthetic medium with $(NH_4)_2SO_4$ as the sole nitrogen source do not undergo derepression. They proposed that this failure to derepress (at low pH) is due to an effect on transcriptional regulation of the APase structural genes. The expression of this enzyme involves at least five regulatory genes: *PHO4* (*PHOD*), *PHO2* (*PHOB*), *PHO81* (*PHOS*), *PHO80* (*PHOR*), and *PHO85* (*PHOU*). Mutations in *PHO4*, *PHO2*, and *PHO81* block derepression of APase, whereas mutations in *PHO80* and *PHO85* result in constitutive enzyme synthesis. In their original regulatory control model,

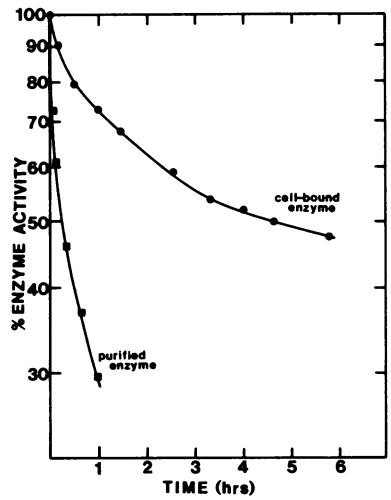

FIG. 9. Kinetics of APase inactivation at pH 3.0. Data from Fig. 7 and 8 for enzyme and cells incubated in $(NH_4)_2SO_4$ minimal medium.

Toh-e et al. (19) proposed that these regulatory genes control structural gene transcription by mechanisms similar to operator control circuits in a bacterial operon. Sequentially, P_i is an inducer for the expression of the *PHO80* and *PHO85* (repressor) genes, which are under the control of *PHO81*. The *PHO80* and *PHO85* gene products cooperatively form a repressor that prevents expression of *PHO4*, a positive regulator of structural gene transcription, by binding to an adjacent control region (operator), the *PHO82* locus. In the absence of P_i, *PHO80* and *PHO85* are not expressed, allowing for production of the *PHO4* product and, subsequently, transcription of the structural gene. However, low pH (blocked derepression) and temperature-shift experiments using temperature-sensitive *PHO2* and *PHO81* mutants (17) suggested revision of this model. Growth at the permissive temperature at low pH followed by a shift-up in pH allowed for derepression at the permissive temperature, but derepression failed to occur when the culture was simultaneously shifted to a nonpermissive temperature. This suggested that the *PHO2* and *PHO81* gene products were concerned with structural gene expression at or after the regulatory step sensitive to low pH (17). As both *PHO4* and *PHO81* are functional at low pH for alkaline phosphatase expression (17, 18, 20) but not APase, Toh-e and co-workers argued that *PHO81* must be directly involved with the function of *PHO4* and *PHO2* rather than in an early stage of the genetic system, as proposed in the original model. In their revised model (17), all regulatory genes are expressed constitutively and function simultaneously. Toh-e et al. proposed that regulatory elements encoded by these regulatory genes are subunits of a protein aggregate that in the absence of P_i promote active transcription of the APase and alkaline phosphatase structural genes. In contrast, in the sequential model, *PHO80* and *PHO85* are expressed only during growth in high-P_i medium and *PHO4* is expressed only during growth in low-P_i medium. Based on data of a different nature, a similar "protein aggregate" mechanism has been proposed for the regulation of the inducible galactose utilization pathway enzymes in *S. cerevisiae* (10, 11).

In this work, we have confirmed that a block in derepression of APase occurs in $(NH_4)_2SO_4$ synthetic medium. By examining the growth parameters leading to this block in derepression, we have defined a rather complex phenomenon involving medium composition and pH. However, in contrast to the findings of Toh-e et al. (16), we have shown that under growth conditions which fail to derepress APase, the enzyme is readily inactivated, an observation consistent with a previous report by Heredia et al. (7) of enzyme instability at low pH. The mechanism of this inactivation at low pH and the mechanism by which some media components provide protection, however, remain unknown.

More significantly, though, we have eliminated the possibility of a disturbance in structural gene transcription as an explanation for this phenomenon. Depletion of P_i during growth in $(NH_4)_2SO_4$ synthetic medium does lead to production of APase mRNA synthetically active in vitro. These results, therefore, negate the pH argument of Toh-e et al. (17) for a revised model for APase regulation.

Other very recent data of Toh-e et al. (16) have also suggested a revision of the sequential regulatory control model. Fine-structure mapping of the *PHO82-pho4* locus demonstrates that the *PHO82* site does not lie adjacent to *PHO4*, as would be expected if it were a classical operator locus, but rather maps to a narrow region located within the *PHO4* gene. This does not negate a regulatory role for the *PHO82* locus in control of *PHO4* transcription, but this revised model would readily explain the mapping data reported by Toh-e et al. if the *PHO82* site codes for a site within the *PHO4* protein involved in subunit interaction with other regulatory factors. Our primary interest in studying the regulation of APase is to understand the nature of the interplay between these multiple positive and negative regulatory genes. In further studies we hope to use the experimental approach adopted here to determine whether these regulatory genes are constitutively expressed. If *PHO2* and *PHO4* are expressed during repres-

sion, then the original sequential regulatory control model will require revision, and some type of direct molecular interaction would most probably be involved.

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